Ethanol Production from Biomass

Robert Bialozynski
Pulaski FFA Chapter
Ag. Science Fair

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Abstract

The purpose of this project was to experiment and decide if small-scale ethanol production from biomass is scientifically feasible by creating experiments to produce ethanol as simply as possible. Then changes were made in problem areas to establish the greatest possible efficiency for the simplest form of ethanol production from biomass.
Procedures used begin with making large quantities of potato dextrose agar (PDA) from scratch and then inoculating PDA agar plates with the freeze-dried stock culture of *Trichoderma Reesei* QM 6a, purchased from ATCC. After growth was established on five PDA agar plates, more were inoculated from the established growth and eventually slants were made as stock cultures. Next, small amounts of potato dextrose broth were inoculated and growth was built up in larger broth batches until about 10% of the fermentation batch was reached. The enzyme production time was spanned over variables to let the *T. reesei* produce a sizeable amount of cellulose and determine exactly how much time was needed for adequate enzyme production.

The enzyme/broth was then dumped onto waste paper pulp and allowed to sit for 0-144 hours of substrate hydrolysis at 25C to determine how much time, if any, was needed to let the cellulase hydrolyze the biomass with out simultaneous fermentation. Then highly active yeast was added to the batch to ferment the sugars hydrolyzed from the paper pulp by the cellulase produced by the *Trichoderma Reesei*.

Data was collected on the variables of enzyme production time, biomass hydrolyzation time without simultaneous fermentation, co fermentation time, and percent ethanol produced from each trial. It was concluded that *Trichoderma Reesei* strain QM 6a is more than adequate for production of cellulases in a simplified small-scale production. Also concluded, is that 168 hours of enzyme production is optimal under current conditions, and that no additional time is needed for substrate hydrolysis before the yeast is added. Ethanol production through this method is efficient and timely enough to be considered a feasible base for individual use and future research of production. However, the economical cost effectiveness wasn’t determined in this study since the focus was on production logistics.

Introduction

As an alternative to accepting the high oil prices from the Middle East and pollution of gasoline use, biotechnology has created a clean burning - potentially inexpensive, alternative that can be produced from the agricultural bounty of our country. This alternative is fuel ethanol, which is currently fermented from carbohydrate rich crops such as corn. However, not enough ethanol can be produced from surplus corn to meet America's growing transportation fuel needs with ethanol as the primary fuel. The solution is hydrolyzing cellulosic biomass, of which, 100 billion tons are grown annually throughout the world. Biomass includes trees, prairie grasses, alfalfa, straws, and even seaweeds. Past processes for hydrolyzing biomass required large amounts of highly concentrated sulfuric acid. Unfortunately, that wasn't cost effective, efficient, or environmentally safe. Recent advancements in biotechnology have genetically modified the fungus Trichoderma Reesei to produce large amounts of extracellular cellulase, the three-part enzyme that hydrolyzes the polysaccharide cellulose, found in biomass, into xylose and fermentable glucose. Yet, the cellulose in raw biomass is incased in lignin that has to be removed with acids and a liquid hot water treatment before any cellulose hydrolyzation. Lignin removal is only feasible when done industrially in large amounts. Therefore, deligninified or low lignin materials such as recyclable paper are needed for efficient small-scale production, which is the focus of my project.

Since there isn't a great number of large biomass ethanol plants in operation, small-scale production would be beneficial if done by a developing country, local municipality, or an individual at home. The small-scale operations could utilize waste paper, cardboard, lawn clippings, leaves, and agricultural wastes to subsidize the fuel use of the community or a household. Plus the operations would be relatively inexpensive to build and simple to run and maintain as well as having the added benefits of flexibility in structure, seasonal product use, expansion, and production amount. This experiment is working towards the applicable data of such a small-scale production of ethanol. The results of my current

experiments approximately predict what materials, resources, time, and processes are required to produce ethanol in the simplest fashion while maintaining industrial practicability.

Review of Literature

Most of the literature and information has actually come from typing in keywords on Google.com such as "*Trichoderma Reesei*", "cellulose", or "biomass hydorlyzation". I would then read through as many of the search results as needed until I found the information I wanted. Although this is a highly unorthodox method of research, it worked unbelievably well for a number of reasons. First of all, there isn't any related literature to my project at any local libraries. Second, all published information on the Internet is extremely specific to one area of the biomass conversion process so I could simultaneously look up several documents at once to help piece all the information together or to help me understand any terms, processes, and measurements that I was unfamiliar with. Lastly, some of the most valuable sources were only represented by an abstract or conclusion of the whole experiment. If the information related to my inquiry, I would find the names and e-mail addresses of the head scientist that submitted the summary and contact them to hopefully receive the full PDF file on their research or ask them for specific information.

When contacting other scientists, I was professional and always showed my gratitude for any help, which was the key to my success. This became an invaluable resource and significantly sped up the information gathering process. The primary work that I used in designing my experiment was a 300-page PDF file I received from Dan Schell, an employee at the Department Of Energy (DOE). I contacted Mr. Schnell after reading an article he published on the experimental *Trichoderma Reesei* hydrolyzation of solka floc (deligninified pine pulp) to observe which strain modification of *T. reesei* worked best. The information he sent me was the actual result of a recent project between NREL (National Renewable Energy Laboratory), branches of the DOE, and independent biotechnology companies including Genencor. This project produced the most modern information on biomass conversion for ethanol production and a detailed design for a

large-scale ethanol plant. (3) The findings of their research began with the shipping of the poplar wood chips and covered everything to the selling of the ethanol at $1.33 a gallon. Information on each step in the process included equipment design, cost estimation, and the scientific importance. This was the ultimate design and resource, but for my project I had to first understand each major step in the process, and then scale down each process to make it as simple and inexpensive as possible in making it feasible for my research and for small-scale production. In essence, I was working backwards to redesign everything on a simpler scale.

Another important source was an article published in 1983 that explained the process of converting biomass into simple sugars using nothing more than sulfuric acid and lime. It also stressed the need for a more cost effective process that included the use of three different cellulolytic enzymes produced from strains of *Trichoderma viride*. This gave me a bearing on how far biomass hydrolyzation has come in only 20 years since only moderate amounts of sulfuric acid are needed today and since *T. viride* has been modified to create *T. reesei*, which now produces all three cellulase enzymes itself. (2)

My third major resource was ATCC (American Type Culture Collection), the biological resource library that I purchased the *T. reesei* from. They have provided the recipe to make potato dextrose agar from scratch, which reduces costs. Plus, they provided links to sources that explain general *T. reesei* growth and provided information on the *Trichoderma* family, which helped me understand the unfamiliar growth patterns of my cultures. (1)

The fourth and last major resource was the documented study of cellulase production of mutated *T. reesei* strains. This study was instrumental in my understanding of the three-part cellulase enzyme. It also informed me what products are made during cellulose hydrolyzation and that more fermentable sugars are released other than glucose. (4)

Materials

- Autoclave
- Petri dishes
- Culture tubes
- Inoculating loop
- Bunsen burner
- Box of matches
- Distilled water
- Small bag of potatoes
- Bag of cane sugar
- Agar powder
- Hot plate
- Incubator
- Masking tape
- Aluminum foil
- 8, 1L flasks
- 8, 250mL flasks
- Safety goggles
- 2, 2L beakers
- 2, 1L beakers
- Paper towel
- Recyclable paper
- Cardboard
- Potato peeler
- Kitchen knife
- *Trichoderma Reesei* culture QM 6a
- Refrigerator
- Gram scale
- Scale dishes
- 10mL graduated cylinder
- Computer
- Notebook
- Writing utensil
- Airlock stoppers
- Airlocks
- Cheese cloth
- Sulfuric acid
- Packet of turbo yeast
- Computer with internet access

Procedure

1. Contact ATCC to obtain certification papers if not already a customer.
2. Certify a laboratory as a biosafety level 1 laboratory by completing the certification papers provided by ATCC.
3. Purchase *Trichoderma Reesei* culture QM 6a from ATCC, product number 13631.
4. Autoclave 5mL of distilled water and keep it in an enclosed vial.
5. Upon receiving the freeze-dried culture, follow the provided instructions in removing the freeze-dried pellet and inserting it into the 5mL-distilled water.
6. Let the suspension rehydrate in the close vial for 24 hours at room temperature (25C).
7. While the culture is rehydrating, make a 1L batch of potato dextrose agar using the recipe provided with the culture.
8. To make the agar, peel four good sized potatoes, cut off 300g of thin potato slices, boil the potato slices in 500mL of water until cooked, strain the liquid through a cheese cloth into a 2L beaker, add distilled water to the broth until it is 1L, add in 20g of glucose and 15g of agar, heat the broth until it is boiling, then separate the 1L into four 200mL beakers, cover them with foil, and autoclave at 121C for 40 minutes.
9. Soon after the autoclaving is completed, aseptically pour out one of the 200mL beakers into sterile disposable petri dishes; make sure to pour the dishes rather thick.
10. Let the agar cool, and then turn the dishes over to reduce condensation on the lid.
11. When the rehydration period is complete, use the eyedropper to aseptically inoculate each petri dish with a few drops of the suspension.
12. Place the inoculated dishes in the incubator and store the extra agar and any left over suspension in a refrigerator at 4C.
13. Set the incubator to 25C.
14. Allow growth for about 48 hours.
15. Select the culture(s) with the densest growth and inoculate more PDA plates from that culture. If the *T. reesei* grows green spores or ferments the agar yellow this is normal of denser cultures.

16. Once positive *T. reesei* growth is established, use the left over PDA to make slant cultures for long-term storage.
17. Make a 1L batch of potato dextrose broth using the PDA recipe used before; just exclude the 15g of agar.
18. Fill 8 vials with 10mL of broth in each one; cover them and autoclave with the rest of the broth.
19. Aseptically inoculate each of the vials by using an inoculating loop. Take each loop from the same Petri dish.
20. Put the vials in the incubator at 25C for 24 hours.
21. Fill 8, 250mL beakers with 100mL of both; cover with foil, and autoclave them.
22. Let the broth cool to room temperature, and then add the 24-hour-old 10mL cultures to the 100mL broths. One vial per beaker.
23. Put the now inoculated beakers in the incubator at 25C for 120 hours.
24. Gather the biomass (recyclable paper) and measure out 6 grams. Amounts needed may change if other types of biomass are used
25. Put the 6 grams of biomass in a standard blender and add in 750mL of distilled water and blend well for about 30 seconds.
26. Pour the biomass slurry from the blender into a 1L flask.
27. Repeat steps 23,24, and 25 until all eight flasks are filled.
28. Autoclave the flasks at 212C or add in campden tablets (sodium metabisulphate) to sterilize the slurry.
29. Let the flasks cool to room temperature or sit loosely covered for 24 hours (if tablet is used) and then pour the inoculated broths into the flasks.
30. Then add in 20g of turbo yeast into each flask and top the flasks with water air locks.
31. Put the flasks in an incubator at 20-30C.
32. Record data on the fermentation observed by studying the growth of the yeast and the rate at which CO2 is being produced and can be visually seen bubbling out of the air lock.

Results

Chart 1

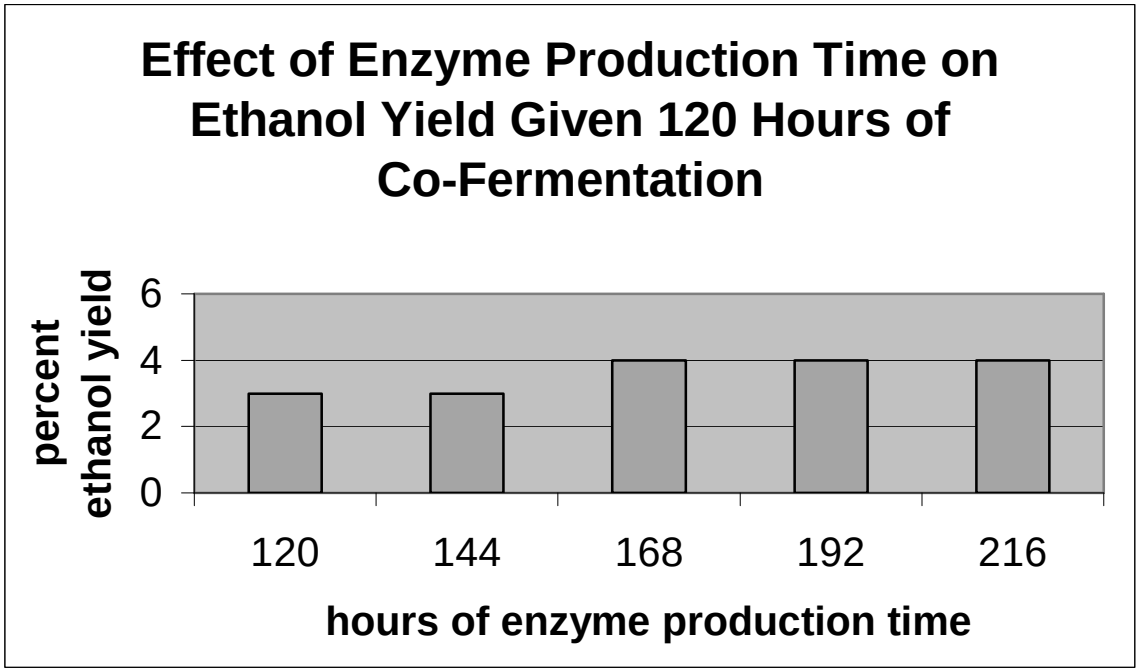
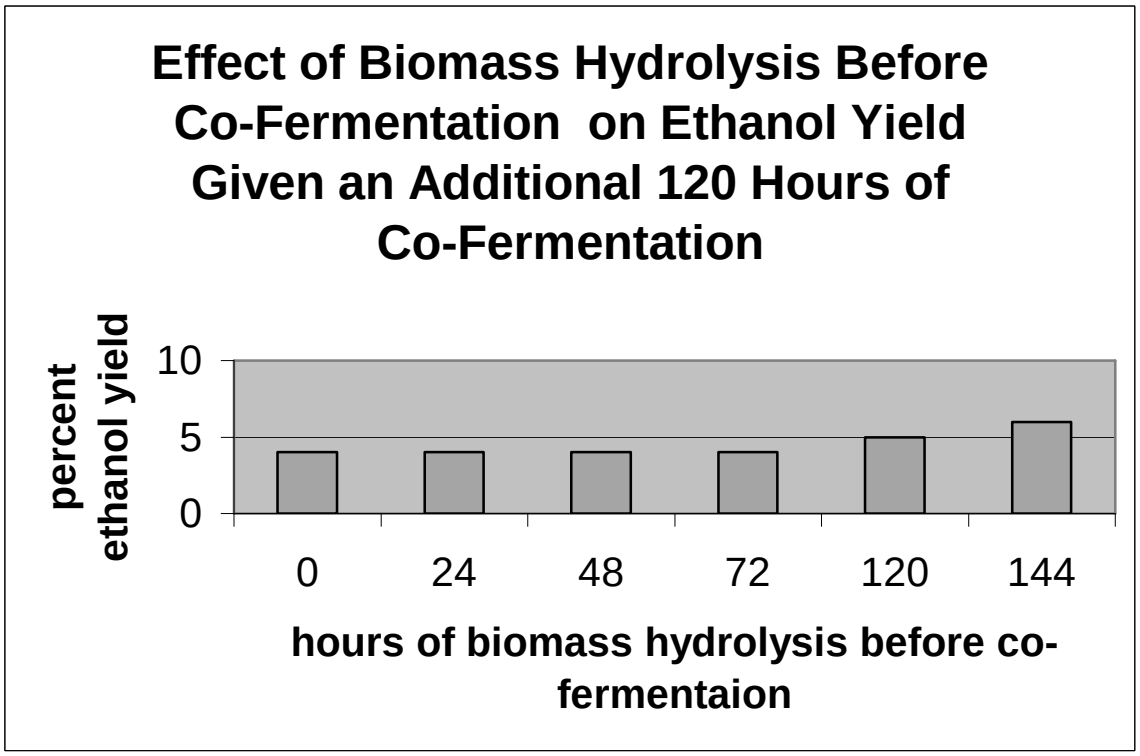


Chart 2



Discussion and Conclusion

Given the data I collected from my experimentation, it can be concluded that the study was a success in establishing that small-scale hydrolyzation of simple biomasses and co-fermentation of ethanol can be achieved in a simple and timely manner. This was proven given the simplicity of a mere 4-stage process with:

stage 1 being the broth enzyme buildup
stage 2 the preparation and sterilization of the biomass slurry
stage 3 the co-fermentation
stage 4 the distillation.

I project that outside of a lab setting these four stages can be easily changed to a continuous flow process instead of the experimented batch system I used. Therefore, more than one line can be run at a time giving a greater output, if desired, along with less required labor. I have also achieved a timely manner when running experimental batches so it can be assumed that a batch can be completed within a week.

I have also concluded that no matter the maturity, density, or sporeous stage of the *Trichoderma reesei*, it has the ability to produce an adequate amount of cellulase to effectively hydrolyze deligninified or processed biomasses. The enzyme batches produced from all three types of *T. reesei* that I cultured showed considerable evidence of cellulase production and all had the same hydrolyzation vigor given visual observations of the yeast fermentation and the total percent ethanol produced which is directly proportional to the glucose hydrolyzed by the cellulase. Another conclusion I reached is that a 168-hour enzyme production period is optimal for allowing the *T. reesei* to produce an adequate amount of cellulase. This was confirmed by the 4% ethanol yield given 120 hours of co-fermentation.

Further concluded, is that the 10% build up for the broth batches over the 168 hour time period was gradual enough to allow the *T. reesei* to grow throughout the added broth while producing enough cellulase. This is reinforced by the data proving that enzyme

hydrolysis time before fermentation did not increase the ethanol yield. Thus proving that there was plenty of enzyme secreted in the 10% build up batches over 168 hours.

The spike in percent ethanol produced at the end of Chart 2 doesn't necessarily suggest that 144 hours of biomass hydrolyzation before fermentation is most favorable. The data is actually misleading for that chart because the 144 hours allowed for hydrolyzation and the 120 hour of co-fermentation total a combined 260 hours of biomass hydrolyzation. Therefore, a 2% greater yield would be expected compared to the 120 hours of hydrolyzation during fermentation from Chart 1.

I consider the study a success with the main point proven of simple biomass conversion and ethanol fermentation attainable by an individual or possibly a municipality or developing country with this process. However, the major flaw in the concept is that small-scale production isn't cost effective enough to out weigh the current prices of gasoline and ordinary cellulolytic waste disposal. In order to make accurate adjustments in making the process more efficient to improve cost effectiveness, more data needs to be examined in a few target areas of the process. Those target areas include measuring the exact cellulase production in the broth stage under a range of conditions and timetables. Another key factor is the exact rate at which the enzyme hydrolyzes the biomass to determine exactly how much cellulase is needed per batch so none goes to waste and no excess time is spent. Plus, it would be helpful to know how much fermentable sugars are given per lb. of specific biomasses and to understand how much ethanol is given per lb of biomass, under optimal conditions, and if all simple sugars including the xylanases are fermented by the yeast strain used.

Plus, the overall process can be redesigned and extremely more cost effective once a ligninase enzyme can be massed produced to hydrolyze the lignin that obstructs the cellulases from breaking down the cellulose strains.

Unfortunately, none of these target areas could be scientifically studied in my experiment due to a lack of equipment. First, the exact cellulase production can't be monitored

because the filter paper used to measure the filter paper units (FPU) of enzyme couldn't be ordered. Also, the exact rate at which the enzyme hydrolyzes the biomass couldn't be measured because sensitive CO2 monitors needed to measure this couldn't fit the limited budget. Plus, the amount of fermentable sugars released can't be measured since a hydrometer reading is inaccurate due to the other impurities in the solution and a glucose strip won't work because there are other sugars produced and the live *T. reesei* consumes some anyway.

Future plans for this study include hydrolyzing and fermenting larger batches of biomass for fuel ethanol that I will then use in my car. I also plan on using my double reflux still to obtain a slightly higher yield from each batch. Plus, I am looking forward to experimenting with other biomasses such as grass clippings. Lastly, I have discussed my research with professors at UW River Falls in hopes of continuing my project with their assistance.

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Mr. Seth Reuter, high school Chemistry Chair, who assisted me in understanding the chemical reactions of the biomass conversion and provided me with the sulfuric acid needed in pre-treating heavier biomass and all the distilled water I needed.

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Green Bay Converting, a paper converting company where my father is employed, donated the rolls of paper that I experimented on.

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